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Developmental and hormonal regulation of α_{2u}-globulin gene transcription

(mRNA abundance/*in vitro* transcription in isolated nuclei/gene expression)

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ABSTRACT **Hepatic α_{2u}-globulin protein and RNA levels are under developmental and complex multihormonal control. The present studies directly evaluate the degree to which this regulation is transcriptional. α_{2u}-Globulin transcription was determined by measuring nuclear runoff RNA *in vitro*, and tissue α_{2u}-globulin mRNA levels were measured by dot blot hybridization. These studies reveal that (*i*) in male rats the transcriptional rate of the α_{2u}-globulin genes increases during postnatal development; (*ii*) no α_{2u}-globulin transcription is detectable in hepatic nuclei derived from hypophysectomized rats; (*iii*) growth hormone and glucocorticoid are both absolutely required, and glucocorticoid can replace androgen for α_{2u}-globulin gene transcription in the livers of hypophysectomized male rats; and (*iv*) chronic treatment of mature male rats with estrogen results in a progressive decrease in the hepatic transcription of α_{2u}-globulin genes. In all instances changes in the transcriptional rate of α_{2u}-globulin genes paralleled the tissue level of α_{2u}-globulin RNA. Thus transcriptional control predominates in regulating hepatic α_{2u}-globulin RNA levels.**

α_{2u}-Globulin, a protein of molecular weight 18,700 (1), is synthesized in the liver, secreted into the blood, and excreted in the urine of male rats (2). α_{2u}-Globulin represents 1–2% of total protein synthesis in the liver (3). This protein is absent or marginally detectable in the liver and urine of female and immature male rats (4); its hepatic synthesis increases in the male rat at puberty, reaching peak levels at about 9–12 weeks of age and decreasing to nearly undetectable levels in senescence (5). The hepatic synthesis of α_{2u}-globulin is under multihormonal control; androgen, glucocorticoid, thyroid hormone, insulin, and growth hormone are required for the excretion of normal levels of α_{2u}-globulin by adult male rats (4, 6, 7). Estrogen administered to mature male rats dramatically inhibits α_{2u}-globulin synthesis (8), whereas androgen administered to ovariectomized female rats induces its synthesis (3). We have recently reported that α_{2u}-globulin is also synthesized in the submaxillary salivary gland (9) and in the extra-orbital lachrymal gland (10).

α_{2u}-Globulin is encoded by a multigene family of approximately 20 highly homologous members (11, 12). At least four of these α_{2u}-globulin genes are expressed in the liver (1, 12), and different subsets of α_{2u}-globulin genes are expressed in the salivary and lachrymal glands (9, 10). Thus, the α_{2u}-globulin system provides an interesting opportunity to study processes underlying hormonal, developmental, and tissue-specific gene expression. Toward this end, we have cloned and sequenced one α_{2u}-globulin gene and a number of hepatic (1, 12), salivary gland (9), and lachrymal gland (unpublished data) cDNAs.

In the case of developmental (13, 14) and endocrine (3, 8, 14–20) control, the *in vivo* rate of synthesis of α_{2u}-globulin and its urinary secretion are paralleled by corresponding alterations in the hepatic level of its mRNA as measured by heterologous *in vitro* translation and hybridization. These studies indicate that the site of action of these controls is pretranslational (for review see refs. 21 and 22). *A priori*, the modulation of a tissue mRNA level might be a consequence of an altered rate of gene transcription, altered stability of the primary transcript or processing intermediates, or differential mRNA turnover (23). It is of import to ascertain which of these processes actually regulate the tissue level of α_{2u}-globulin mRNA. In this report, we have directly measured the relative transcriptional rate of α_{2u}-globulin genes in isolated liver nuclei derived from rats in different functional states. These studies demonstrate that developmental and hormonal control of hepatic α_{2u}-globulin RNA levels is predominantly by selective regulation of the transcription of α_{2u}-globulin genes.

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EXPERIMENTAL PROCEDURES

Animals, Hormone Supplement, and Recombinant DNA. Intact and hypophysectomized Sprague–Dawley rats (Charles River Breeding Laboratories) were used in all experiments. Evaluation of complete hypophysectomy and duration and dosage of hormonal supplementations were as described earlier (14). For chronic estrogen administration male rats, 280–320 g, were used and 17β-estradiol (Sigma), 2 mg/kg of body weight, was injected intraperitoneally as described (8). The clones, 207-4, containing the entire α_{2u}-globulin gene, and pSGII, containing α_{2u}-globulin cDNA, have been described in detail (9, 12).

Transcription in Isolated Nuclei. Nuclei were isolated from rat liver essentially as described by Mulvihill and Palmiter (24). The procedure for *in vitro* nuclear transcription, isolation of RNA, and hybridization is similar to that described by McKnight and Palmiter (25). Nuclei 3–6 $\times$ 10^8 were incubated, usually in triplicate, for 45 min at 26°C in a 100-μl reaction mixture containing 16% (vol/vol) glycerol, 20 mM Tris·HCl at pH 8.0, 5 mM magnesium chloride, 150 mM potassium chloride, 0.4 mM each of ATP, GTP, and CTP, and 100 μCi of [α-^{32}P]UTP (100–400 Ci/mmol, Amersham; 1 Ci = 37 GBq). Nuclear RNA was freed of DNA and protein, total trichloroacetic acid-precipitable radioactive material was measured, and aliquots were taken for filter hybridization. For this procedure, 0.2 μg of DNA from 207-4, pSGII, or pBR322 was immobilized to 7-mm nitrocellulose filters, hybridization was carried out at 45°C for 3–4 days, and filters were washed and treated with RNase A and RNase T1. The major deviation from the procedure described in ref. 25 was that the filters were given an additional stringent wash in 0.15 M NaCl/2 mM EDTA/10 mM Tris·HCl, pH 7.5/0.1% sodium dodecyl sulfate at 65°C for 30 min. The filters were air dried and their radioactivities were determined in 7 ml of Econofluor (New England Nuclear). The scintillation counter (1215 Rackbeta from LKB) had a background of 14 cpm and each sample was counted at least for 30 min.

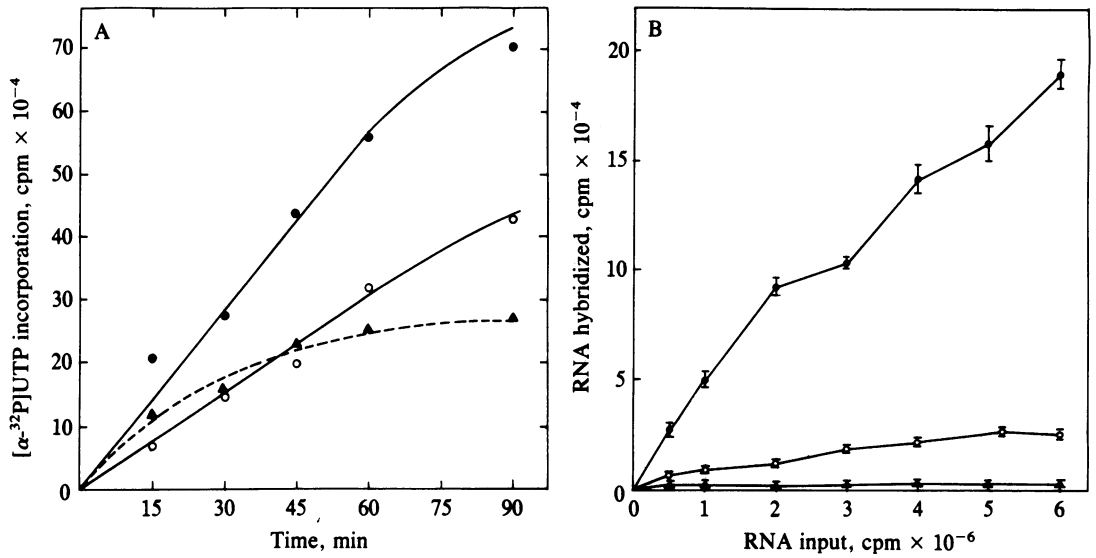

FIG. 1. Time course of RNA synthesis in isolated liver nuclei and hybridization of α_{2u}-globulin RNA transcripts to a cloned α_{2u}-globulin gene and cDNA. (*A*) Nuclei were incubated in the presence (○) and absence (●) of α-amanitin at 2.5 μg/ml. Aliquots were removed at the indicated times and acid-precipitable radioactivity was determined. α-Amanitin-sensitive RNA polymerase II activity (▲) was calculated. (*B*) Newly synthesized nuclear RNA was hybridized to 0.2 μg each of α_{2u}-globulin genomic plasmid p207-4 (●), cDNA plasmid pSGII (○), or wild-type pBR322 DNA (▲) and RNase-resistant radioactive material was measured. Bars indicate ± SEM.

RNA Isolation and Blots. Liver RNA was extracted by the guanidine thiocyanate method (26). Each RNA sample was checked for integrity by blot hybridization analysis as described earlier (14). Relative amounts of α_{2u}-globulin RNA sequences were measured by applying 0.25–1.0 μg of total RNA to nitrocellulose paper with a dot blot template (Minifold from Schleicher & Schuell) and were hybridized to an excess of nick-translated full-length α_{2u}-globulin cDNA as described (9, 10).

RESULTS AND DISCUSSION

RNA Polymerase II Transcription and DNA-Excess RNA Hybridization. When isolated nuclei are incubated in the presence of α-amanitin (2.5 μg/ml), the incorporation of [α-^{32}P]UTP is inhibited by about 50%. This RNA polymerase II-mediated incorporation of [α-^{32}P]UTP is essentially completed by 45 min (Fig. 1*A*). Parallel experiments indicate that this level of α-amanitin prevents 98% of the α_{2u}-globulin gene transcription (data not shown). Fig. 1*B* depicts the linear range of α_{2u}-globulin RNA, newly synthesized by isolated liver nuclei, hybridizing to the 0.2 μg of either α_{2u}-globulin genomic plasmid or cDNA plasmid. There is little hybridization to pBR322 itself and, as expected, the extent of hybridization of the newly synthesized α_{2u}-globulin RNA transcripts to its gene is several times greater than to its cDNA (Fig. 1*B*). Furthermore, 3 kilobase pairs of 5′ flanking sequences of genomic clone (207-4) do not hybridize to any transcripts, and transcripts from isolated nuclei of nonexpressing tissues such as kidney do not hybridize to 207-4 (data not shown).

Age- and Sex-Dependent Control of α_{2u}-Globulin Gene Expression. α_{2u}-Globulin RNA sequences are undetectable in the livers of prepubescent male rats; they appear initially at the onset of puberty, reach peak levels at about 9–12 weeks of age, and decrease to nearly undetectable levels in senescence (13, 14). To determine whether the appearance of α_{2u}-globulin mRNA at puberty is due to increased transcription of these genes, we measured the rate of transcription in

Table 1. Age- and sex-dependent control of hepatic α_{2u}-globulin transcription

Age, days	Sex	Total RNA input,* cpm × 10^{-6}	RNA hybridized, cpm: 207-4	pBR322	207-4 − pBR322	α_{2u}-Globulin transcription rate,† ppm	*P* value for transcription rates
				Experiment I			
25	Male	2.6	110 ± 10 (5)	95 ± 10	15 ± 3	6 ± 1	—
34	Male	2.6	82 ± 4 (5)	66 ± 6	16 ± 3	6 ± 1	—
44	Male	2.6	187 ± 6 (5)	70 ± 2	117 ± 6	45 ± 2	$P < 0.001$ vs. 34 days
54	Male	2.6	590 ± 25 (6)	85 ± 7	505 ± 23	194 ± 8	$P < 0.001$ vs. 44 days
64	Male	2.6	637 ± 28 (6)	61 ± 4	577 ± 28	222 ± 11	$P < 0.10$ vs. 54 days
74	Male	2.6	567 ± 23 (5)	65 ± 8	502 ± 15	193 ± 5	$P < 0.05$ vs. 64 days
				Experiment II			
65	Male	3.0	839 ± 55 (10)	63 ± 6	776 ± 55	259 ± 19	$P < 0.001$ vs. 65-day-old females
65	Female	3.0	122 ± 4 (9)	57 ± 9	65 ± 11	22 ± 3	—

For preparation of nuclei three or four livers were combined. Nuclei were incubated in the presence of 0.1 mCi of [α-^{32}P]UTP for 45 min at 26°C. Nuclear RNA was extracted, freed of protein and DNA, and hybridized in duplicate to excess DNA (0.2 μg) of pBR322 or pBR322 subclone 207-4 containing α_{2u}-globulin genomic sequences bound to nitrocellulose filters; the RNase-resistant radioactive material was measured. Values are expressed as mean ± SEM; numbers in parentheses indicate number of hybridization values. Significance of differences between transcription rates was calculated by using Student's *t* test.

*Input is the amount of total radioactive RNA transcription product subjected to hybridization.

†The average transcription rate is defined as ppm of input RNA that is hybridized to the α_{2u}-globulin genomic subclone, 207-4.

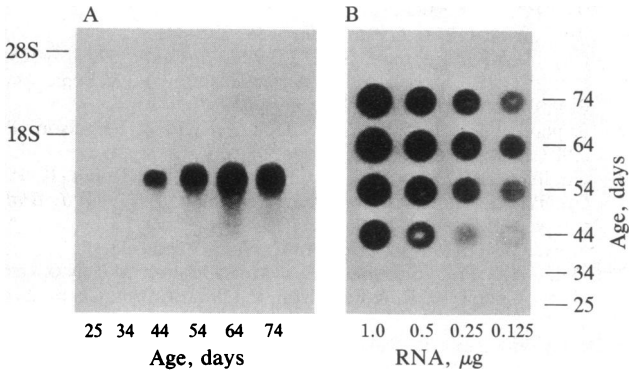

FIG. 2. Developmental profile of male rat hepatic α_{2u}-globulin RNA. (*A*) Electrophoretic blot analysis: Ten micrograms of total RNA derived from rats of indicated ages was electrophoresed through a 1% agarose/6% formaldehyde gel, transferred to nitrocellulose paper, and hybridized to nick-translated full-length α_{2u}-globulin cDNA. (*B*) Dot blot analysis: Total RNA was applied to nitrocellulose paper. The bound RNA was hybridized to an excess of nick-translated α_{2u}-globulin cDNA overnight at 42°C and the blot was washed to remove nonhybridized probe. The nitrocellulose filter was then dried and exposed to x-ray film for 16 hr.

liver nuclei derived from animals of various ages. These studies show a very low hepatic α_{2u}-globulin transcription in prepubescent male rats (Table 1). Transcription of these genes increases dramatically at puberty and reaches peak levels in 64-day-old animals. At all ages, the relative levels of α_{2u}-globulin mRNA, as measured by electrophoretic and dot blot hybridization analysis (Fig. 2) are in essential agreement with their relative rates of transcription. Thus, ontogenetic control of α_{2u}-globulin synthesis seems to be predominantly transcriptional.

Livers of female rats do not detectably synthesize α_{2u}-globulin, nor do they contain measurable level of functional

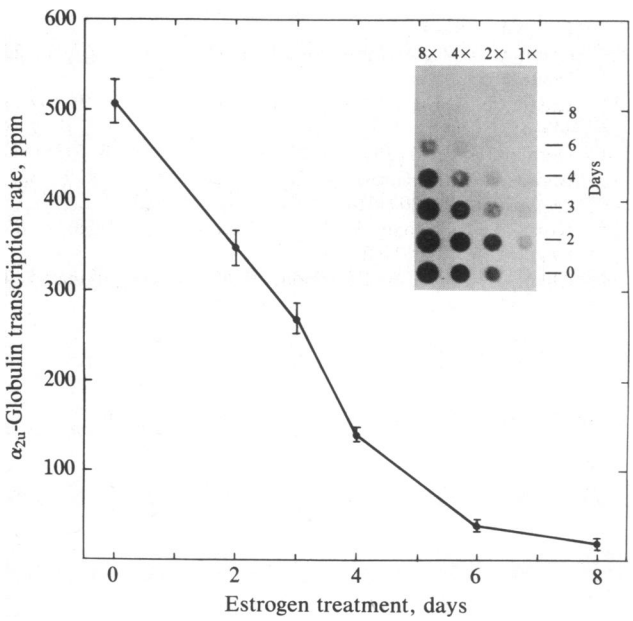

FIG. 3. Effect of chronic administration of estrogen on the rate of transcription of hepatic α_{2u}-globulin genes in isolated nuclei. Mature male rats were injected intraperitoneally daily with 17β-estradiol (2 mg/kg of body weight) and sacrificed at the indicated intervals. Liver nuclei were isolated and transcription was evaluated. Each point represents a mean (±SEM) of nine hybridizations. (*Inset*) Hepatic α_{2u}-globulin RNA levels as determined by dot blot analysis. Conditions for the dot blot are same as in Fig. 2; 1× = 0.125 μg of RNA.

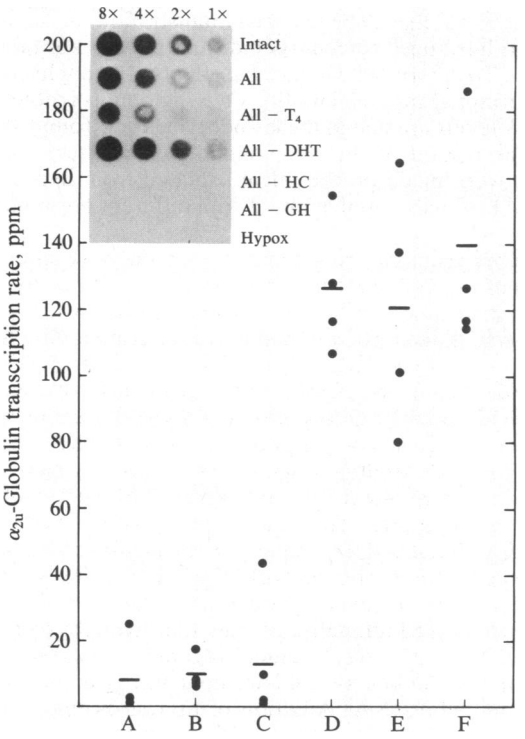

FIG. 4. Effects of hypophysectomy and hormonal supplementation of hypophysectomized rats on hepatic α_{2u}-globulin RNA levels and the transcriptional rate of α_{2u}-globulin genes in liver nuclei. Male rats were hypophysectomized at 48 days of age, rested for the next 20 days, and treated for 12 days with combinations of following hormones: growth hormone (GH; 1 mg/kg, subcutaneous), hydrocortisone acetate (HC; 30 mg/kg, intraperitoneal), dihydrotestosterone (DHT; 2 mg/kg, intraperitoneal), and thyroid hormone (thyroxine) (T_4; 0.2 mg/kg, intraperitoneal). Each point depicts a single hybridization and the bar indicates the mean value of each group. The experimental groups are as follows: A, hypophysectomy (Hypox); B, hypophysectomy plus HC, DHT, and T_4 (All − GH); C, hypophysectomy plus GH, DHT, and T_4 (All − HC); D, hypophysectomy plus GH, HC, and T_4 (All − DHT); E, hypophysectomy plus GH, HC, DHT, and T_4 (All); F, intact controls (Intact). Conditions for the dot blot (*Inset*) are same as in Fig. 2.

(27) or hybridizable (10, 28) α_{2u}-globulin RNA. The present studies indicate a high rate of α_{2u}-globulin gene transcription in hepatic nuclei derived from mature male rats and a very low but reproducible rate of α_{2u}-globulin gene transcription in hepatic nuclei derived from mature females (Table 1). The major urinary proteins (MUPs) in mouse, also encoded by a multigene family, are homologous to rat α_{2u}-globulin (29). Similar sex-related transcription of mouse MUP genes has been reported (30).

Effects of Chronic Estrogen Treatment on α_{2u}-Globulin Gene Transcription. Chronic administration of 17β-estradiol to mature male rats results in a dramatic decrease in α_{2u}-globulin mRNA levels in 6–8 days (8). To evaluate whether estrogenic control of hepatic α_{2u}-globulin mRNA level is mediated transcriptionally, we administered 17β-estradiol up to 8 days and measured nuclear transcription rates. This study indicates a progressive and ultimately essentially complete inhibition of α_{2u}-globulin gene transcription (Fig. 3) without affecting the overall hepatic transcriptional rate (data not shown). This prolonged transcriptional suppression correlates with and presumably is responsible for decreases in the hepatic levels of α_{2u}-globulin RNA sequences (Fig. 3) and α_{2u}-globulin protein (8).

Hormonal Control of α_{2u}-Globulin Gene Transcription. Hypophysectomy of mature male rats results in a complete selective loss of hepatic α_{2u}-globulin synthesis; this can be

reversed by the concurrent administration of androgen, thyroid hormone, glucocorticoid, and growth hormone (14, 17, 21). To determine whether the effects of hypophysectomy and hormonal supplementation on α_{2u}-globulin synthesis and mRNA levels are due to these endocrines controlling the rate of transcription of the α_{2u}-globulin genes, direct measurements were made on the nuclei isolated from hypophysectomized animals supplemented with different hormonal regimens.

Hypophysectomy results in an essentially complete cessation of α_{2u}-globulin transcription (Fig. 4). When hypophysectomized rats were supplemented with growth hormone, androgen, glucocorticoid, and thyroid hormone, the hepatic α_{2u}-globulin transcriptional rate was restored to 75% that of the intact controls. Replacement regimens lacking either growth hormone or glucocorticoid only very weakly induced α_{2u}-globulin gene transcription in the hypophysectomized rats. However, treatment with the hormone regimen lacking only androgen effectively fully restored the α_{2u}-globulin transcriptional rate. The relative steady-state levels of hepatic α_{2u}-globulin RNA sequences, as measured by dot blot analysis, again correlate with the relative transcriptional rates for the respective experimental groups (Fig. 4). The dot blot analysis, additionally, reveals that hypophysectomized rats treated with a replacement regimen containing growth hormone but lacking thyroid hormone have significant levels of α_{2u}-globulin RNA. Addition of thyroid hormone further increases α_{2u}-globulin RNA. Thus growth hormone and glucocorticoid are absolutely required for the hepatic transcription of α_{2u}-globulin genes but androgen can be replaced by glucocorticoid. These transcriptional results are compatible with observations that glucocorticoid reverses estrogenic inhibition of α_{2u}-globulin synthesis (31) and restores normal α_{2u}-globulin levels in the livers of castrated adult males *in vivo* (32) and in cultured hepatocytes *in vitro* (22, 32, 33).

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